Prenatal Diagnosis Using Fetal Cells From the Maternal Circulation

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All current methods of fetal karyotyping are invasive and carry a definite, albeit small, procedure-related risk. Because of this and testing costs, only women older than 35 years who have a greater risk for fetal aneuploidy are currently offered prenatal testing. But this detects only 20% to 25% of fetuses with Down syndrome. It would be a tremendous advance to find a noninvasive technique for prenatal diagnosis that carries no procedure-related risk and could be offered to all pregnant women. We describe a possible technique for noninvasive prenatal diagnosis that aims to identify fetal cells in the peripheral maternal circulation and successfully garner them for prenatal testing.

Early attempts at fetal karyotyping were hampered by inaccurate diagnostic methods and cumbersome cell-counting techniques. Today, improved capabilities of identifying and enriching for fetal cells, coupled with sensitive methods of analysis such as the polymerase chain reaction, bring renewed enthusiasm to this task. Many technical issues, as well as serious questions regarding the test's utility, still exist, however, and must be explored and answered before the capture of fetal cells in the maternal circulation translates into reality for noninvasive prenatal diagnosis.

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At least two reasons exist for the current increased interest in prenatal diagnosis. More women at an advanced maternal age, defined as age over 35 years at the time of delivery, are choosing pregnancy, and these women are at a considerable risk for fetal aneuploidy. In addition, because of the explosion of information in molecular genetics, an increasing number of hereditary disorders are detectable prenatally.

Currently we have three routine ways of obtaining fetal tissue: amniocentesis at 15 to 20 weeks' gestation, chorionic villus sampling at 10 to 12 weeks, and percutaneous umbilical blood sampling past 18 weeks. In addition, experimental invasive fetal sampling methods such as early amniocentesis, late transabdominal chorionic villus sampling, skin biopsy, liver biopsy, and muscle biopsy are being used. Although amniocentesis and chorionic villus sampling are both widely accepted and relatively safe, they are both invasive and carry a definite, albeit small, procedure-related risk. Because of this risk and the cost of testing, only women older than 35 who have a greater risk for fetal aneuploidy are currently offered prenatal testing. Because testing detects only 20% to 25% of fetuses with Down syndrome, finding a technique for prenatal diagnosis that is noninvasive and carries no procedure-related risk would be a tremendous advance that could be offered to all pregnant women. We describe a possible technique for noninvasive prenatal diagnosis that will identify fetal cells in the peripheral maternal circulation and successfully garner them for prenatal testing.

Distinguishing Fetal From Maternal Cells

Three major challenges must be met. First, we must distinguish fetal cells from maternal cells in a process that must be both sensitive and specific. Because the number of desired fetal cells will be small, we must have a way to select for them. By an enrichment process using techniques such as the fluorescent-activated cell sorter, various measures distinguishing fetal cells from maternal cells can be studied and used to concentrate the specimen for fetal cells. Finally, the genetic material in the collected fetal cells will be analyzed with the aid of rapid and sensitive techniques such as in situ hybridization and polymerase chain reaction.

The literature as far back as 1893 contains descriptions of the transplacental passage of fetal cells into the maternal circulation when Schmorl reported finding trophoblasts in the lungs of women dying of eclampsia.[1] Although originally thought to be due to increased uterine

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ABBREVIATIONS USED IN TEXT

FISH = fluorescence in situ hybridization
PCR = polymerase chain reaction

manipulation or maternal trauma, the transplacental passage of fetal cells during the course of normal pregnancy was later reported by many investigators. Douglas and co-workers found large cells with 30 or more nuclei and eosinophilic cytoplasm in the broad ligament veins of women with gestations as early as 18 weeks.[2] These were thought to be examples of syncytiotrophoblasts. Goodfellow and Taylor found syncytiotrophoblasts in the antecubital veins of six of ten women between 8 weeks' gestation and full term.[3] Walknowska and colleagues searched for male fetal lymphocytes by counting five small acrocentric chromosomes and labeling them "XY" cells.[4] They found such cells in 19 of 21 women who gave birth to male infants. Unfortunately, they also found such cells in six of nine women who gave birth to female infants. Schroder and de la Chapelle used quinacrine-stained portions of the Y chromosome to direct their search.[5] Although their results showed an encouraging number of positive-fluorescent cells in women pregnant with male fetuses, implying that passage of lymphocytes into maternal circulation was appreciable, the accuracy of predicting fetal sex was not appreciably better than that obtained by Walknowska's method.

All of these early investigations had a worrisome incidence of false-positive and false-negative results. False-positive results were thought to arise from artifact, nonspecific binding, or homologous areas of fluorescence on parental autosomes. False-negative results may have been due to the nondetection of the variable Y-fluorescent area because it was too small to detect or, more likely, from too few cells being counted to detect reliably the few fetal cells in the maternal circulation. A refinement of this fluorescent technique excluded mothers with bright autosomal fluorescence, which reduced the number of false-positive results, but did little for the unacceptably high percentage of false-negative results.[6]

A major improvement in the method came with the introduction of fluorescently-tagged monoclonal antibodies. A number of monoclonal antibodies raised against antigens on various fetal cells have been used. In 1984, Covone and co-workers used a murine monoclonal antibody, H315, to detect syncytiotrophoblasts in the peripheral circulation of 46 pregnant women.[7] They did not confirm the fetal origin of these cells, however, and other investigators were not able to duplicate their results. Pool and colleagues used the same antibody and did not detect H315+ cells in a quarter of 62 pregnant women tested.[8] A great variability between patients and substantial overlap between pregnant and nonpregnant women were found. Adinolfi reported that most H315+ cellular elements did not react with Y chromosome-specific probes, regardless of whether the fetus was male.[9] He postulated that maternal blood cells adsorbed H315 as they circulated through the placenta.

Mueller and co-workers screened 6,800 monoclonal antibodies to find 5 that appeared to react specifically to a membrane antigen on syncytiotrophoblasts and nonvillous cytotrophoblasts.[10] The labeled cells were then gathered using magnetic beads and proved their fetal origin by polymerase chain reaction (PCR) amplification of a Y chromosome-specific DNA sequence. Of 13 women carrying male fetuses, 12 had accurate prediction of fetal sex by this method.

A number of antigens have been studied on fetal erythrocytes. Fetal erythrocytes have been found in the maternal circulation and are thought to be the result of transplacental bleeding. The fetal circulation is distinguished from the maternal circulation because the former contains a large number of erythroid precursor cells that are nucleated and larger (200 μm^3 compared with 90 μm^3). A number of erythrocyte cell surface antigens have been examined. The first seemingly promising antigen was the transferrin receptor found on nucleated erythrocytes that were actively incorporating the iron transferrin complex. Bianchi and colleagues used a monoclonal antibody against this transferrin receptor to identify fetal erythrocytes, enriched the fetal population using cell-sorting techniques, and confirmed the presence of male (fetal) DNA in six of eight pregnancies carrying male fetuses using the PCR amplification technique.[11] The authors could detect as little as 100 pg of fetal DNA or the equivalent of 15 fetal cells. Other investigators, as well as Bianchi and associates in subsequent unpublished data, have found that the transferrin receptor has limited utility when used by itself. Ganshirt-Ahlert and co-workers used this antibody and the magnetic-activated cell sorter to enrich for nucleated erythrocytes and found that this process detected only 25% of the desired cells.[12] Others have used two surface antigens, transferrin receptor and glycophorin A, in conjunction with other physical cell measurements, size, and granularity, with all being evaluated simultaneously in a multiparameter fluorescence-activated cell sorter.[13,14] Price and colleagues sorted for fetal erythrocytes using this method, performed PCR for a single-copy Y-specific DNA sequence, and correctly identified male sex in 12 of 12 fetuses.[13] Five of six female fetuses were appropriately negative for the Y-specific DNA sequence. It appears that, although the number of fetal cells in the maternal circulation is small, with the help of a combination of specific fluorescent antibodies, cell sorting techniques, and PCR amplification, a correct diagnosis of fetal sex can be made in most cases.

Sporadic cases of aneuploidy also have been accurately detected. Price and associates detected one case of trisomy 18 in a maternal blood specimen taken one week after chorionic villus sampling and one case of trisomy 21 in a maternal blood specimen taken just before chorionic villus sampling.[13] We have seen one case of trisomy 21 in a maternal blood specimen taken two weeks after amniocentesis. A multicenter clinical trial involving thousands of women is needed to systematically study the diagnostic accuracy of fetal cells obtained from maternal blood before this test can be offered clinically.

Fluorescent-Activated Cell Sorting

Basic Principles

The flow cytometer is a sophisticated instrument for studying various physical and chemical properties of particles as they flow single file past an observation point.[15] Biologic particles of various sizes have been studied successfully, including large immune complexes, individual viral particles, liposomes, cellular organelles, bacteria, fungi, chromosomes, eukaryotic cells, cell hybrids, cell aggregates, and multicellular organisms. A number of physical and chemical characteristics can be measured. Structural features such as cell size, cell shape, DNA:RNA content, or cell surface antigens are particularly useful in distinguishing cells in a mixed population. Functional measurements such as membrane permeability, membrane integrity, enzyme activity, or surface charge are also possible. Several variables can be studied simultaneously with multiparameter cell sorters. With the aid of the computer, the characteristics of each cell are compared with preselected characteristics, and once the desired subpopulation of cells is identified, these cells are diverted to form a pool of viable cells that have been enriched for a particular cell type.

Analysis

By using cell-sorting techniques, a mixed population of fetal and maternal cells can be enriched for fetal cells. The degree of enrichment possible varies, depending on the power of the different tests used to distinguish between the fetal and maternal cell populations. Methods of analysis that require minimal amounts of DNA are helpful. For rapid and specific diagnoses using small amounts of DNA, the polymerase chain reaction method is superior. Using PCR, the amount of DNA in one or a few cells can be amplified into a quantity sufficient for prenatal detection. This is done by first denaturing or separating DNA into its two complementary strands. Oligonucleotide primers, or short starting pieces of DNA that are complementary to the desired sequence, are added in such a way that they anneal or bind to their complementary sequences. A DNA polymerase synthesizes a new strand by extending the primer sequence. By repeating these steps, many copies of the segment of DNA between the two primers are made. By using the appropriate primers, this technique can quickly establish the presence or absence of a particular sequence, such as one on the Y chromosome to determine the sex of a fetus that may be at risk for an X-linked disease. Clearly PCR can aid in the prenatal detection of diseases for which the mutation is known—sickle cell disease, cystic fibrosis, β-thalassemia—or in which the sequence at a genetically linked restriction fragment length polymorphism is known—hemophilia A.

How sensitive is the PCR technique? Theoretically it can detect the amount of DNA present in one cell. To test this sensitivity, Adinolfi and co-workers amplified DNA from one cell in cell-free media and were able to obtain a positive result.[16] The same result was obtained when the cell was placed in a background of 3,500 "undesired" cells. One cell of interest in a background of 7,000 cells gave only a weakly positive result, however. One cell among 1×10^5 background cells could not be amplified using one set of primers.

Using nested primers—one set of primers situated inside another set—Lo and colleagues were able to amplify one cell in a background of 1×10^7 negative cells and were thus able to increase the sensitivity of the PCR detection method.[17] Sensitivity was limited, however: false-positive results became a problem when the number of amplification cycles exceeded 20, illustrating the traditional problem of sacrificing specificity for increasing sensitivity.

In Situ Hybridization

Fluorescence in situ hybridization (FISH) is a useful new technique for the rapid detection of cytogenetic abnormalities. This technique uses fluorescently labeled, chromosome-specific probes to identify chromosomal aberrations, both in number and in structure. Because of its technical convenience in using nonradioactive probes and the increased availability of probes that hybridize intensely and specifically to selected chromosomes, this technique promises to have increasing use in the next few years.

Most probes bind to repeated sequences on regions of the target chromosome near the centromere. The fluorescence of the probe allows for a rapid identification of the number of copies and the gross structural makeup of the chromosomes in interphase nuclei. Lucas and co-workers used in situ hybridization to fluorescently stain regions that flank human chromosome 1p to detect structural aberrations involving this region.[18] A successful diagnosis of trisomy 18 has been reported by using amniotic fluid cells hybridized with a repetitive sequence probe that, under highly stringent conditions, is specific for the centromeric region of chromosome 18. Lichter and colleagues reported the successful rapid detection of numerical and structural aberrations of chromosome 21 in metaphase and interphase cells using DNA probes that specifically label the terminal band 21q22.3 by in situ hybridization.[19] Interphase in situ hybridization is especially promising for clinical applications where rapid results are desirable, where cells are difficult to culture in vitro, or where concern exists about the ability to stimulate a representative fraction of cells into proliferation for metaphase analysis.

In situ hybridization can be applied directly to fetal cells isolated from the maternal circulation. Specific repeat probes are currently available for chromosomes 18, X, and Y, and one probe detects both chromosomes 13 and 21. By using two different stains, such as fluorescein (yellow-green) and rhodamine (red), two different chromosomes can be visualized at the same time, thus creating a way to distinguish male fetal cells from maternal cells. Common trisomies can also be rapidly detected. Using the two-color technique, one fetal cell can be detected in 5×10^5 to 5×10^6 maternal cells.

Conclusion

The search for fetal cells in the maternal circulation has been going on for many decades. The tools for fetal cell identification and enrichment have improved considerably, and for the first time we have an extremely sensitive method of analysis in PCR. This may prove to be a crucial difference because fetal cells rarely appear in the maternal circulation. Despite these advances, several major difficulties remain.

First, the problem of false-positive and false-negative results has not been completely solved. To date, no large study has been able to predict the sex of a fetus with complete accuracy regardless of the methods of enrichment or the vehicle for analysis used. Explanations for false-positive results include PCR contamination or circulating male cells from a previous pregnancy. False-negative results could easily arise from loss of the rare fetal cells somewhere in the enrichment process. This becomes a problem in the case of FISH analysis, when scoring a cell as normal or trisomic depends on the proportion of cells counted as trisomic, not just on one cell scored as trisomic. In our experience, as many as 20% of cells can be scored as trisomic in a pure population of normal cells. Because the recovery of fetal cells is sparse at best, there may not be enough fetal cells to count to clearly distinguish a trisomic or aneuploid cell from a normal cell. Hence, although the reports of aneuploid detection from maternal blood are encouraging, they are based on aneuploidy rates as low as 2.8% of cells counted; a large series of unknown specimens should be studied to find out, in a euploid population, whether 2.8% or more of cells would also appear to have an abnormal number of dots purely from technical variability.[20]

Apart from the technical limitations revolving around the detection of a Y chromosome-specific sequence against a maternal background, numerous questions remain that pertain to the usefulness of looking at fetal cells in this manner. Certainly the test could be a noninvasive way to determine fetal sex for X-linked disorders. Beyond this, however, its usefulness may be limited. Detecting a Y chromosome sequence against a negative background of maternal cells is one thing, but detecting point mutations or even large deletions by PCR or FISH may prove impossible. In X-linked or autosomal recessive disorders, the inability to quantitate the dose of abnormal chromosomes present will be a major handicap. Against a background of cells from a woman who is a carrier and who has both normal and abnormal gene sequences, a definitive diagnosis of the fetal genotype by PCR will be difficult. In autosomal dominant disorders, again, the test will be difficult to interpret in all cases where the mother is affected and carries both normal and abnormal sequences in the background against which the fetal cell must be evaluated. Finally, to attain the wealth of information available by invasive methods today, the fetal cells after identification and separation must be viable to provide metaphase preparations for cytogenic studies.

Whether fetal cells cross the placenta into the maternal circulation and, if so, whether they do so in a sufficient number to allow for prenatal diagnosis are questions that have been debated for several decades. Early attempts to answer these questions were hampered by inaccurate diagnostic methods and cumbersome cell-counting techniques. Today we have improved capabilities for identifying fetal cells using a variety of cell surface-specific antigens and flow cytometry techniques to enrich for the fetal cell population in the maternal circulation. The DNA in the few fetal cells selected in this way can then be amplified with sensitive methods of analysis such as the polymerase chain reaction and analyzed with in situ hybridization. Many technical issues, as well as serious questions regarding the test's utility, still exist and must be explored and answered before the capture of fetal cells in the maternal circulation translates into reality for noninvasive prenatal diagnosis.

REFERENCES

1. Schmorl G: Pathologisch-Anatomische Untersuchungen über puerperal Eklampsie. Leipzig, Germany, Vogel, 1893

2. Douglas GW, Thomas L, Carr M, et al: Trophoblast in the circulating blood during pregnancy. Am J Obstet Gynecol 1959; 78:960-973

3. Goodfellow CF, Taylor PV: Extraction and identification of trophoblast cells circulating in peripheral blood during pregnancy. Br J Obstet Gynaecol 1982; 89: 65-68

4. Walknowska J, Conte FA, Grumbach MM: Practical and theoretical implication of fetal/maternal lymphocyte transfer. Lancet 1969; 1:1119-1122

5. Schroder J, de la Chapelle A: Fetal lymphocytes in the maternal blood. Blood 1972; 39:153

6. Schroder J, Tiilikainen A, de la Chapelle A: Fetal leukocytes in the maternal circulation after delivery—I. Cytological aspects. Transplantation 1974; 17:346

7. Covone AE, Mutton D, Johnson PM, Adinolfi M: Trophoblast cells in peripheral blood from pregnant women. Lancet 1984; 536:841-843

8. Pool C, Aplin JD, Taylor GM, Boyd RD: Trophoblast cells and maternal blood (Letter). Lancet 1987; 1:804-805

9. Adinolfi M: Detection of trophoblast cellular elements in maternal peripheral blood using monoclonal antibodies and flow cytometry. Cytometry [suppl] 1988; 3:104-105

10. Mueller UW, Hawes CS, Wright AE, et al: Isolation of fetal trophoblast cells from peripheral blood of pregnant women. Lancet 1990; 336:197-200

11. Bianchi DW, Flint AF, Pizzimenti MF, Knoll JH, Latt SA: Isolation of fetal DNA from nucleated erythrocytes in maternal blood. Proc Natl Acad Sci USA 1990; 87:3279-3283

12. Ganshirt-Ahlert D, Burschyk M, Garritsen HS, et al: Magnetic cell sorting and the transferrin receptor as potential means of prenatal diagnosis from maternal blood. Am J Obstet Gynecol 1992; 166:1350-1355

13. Price JO, Elias S, Wachtel SS, et al: Prenatal diagnosis with fetal cells isolated from maternal blood by multiparameter flow cytometry. Am J Obstet Gynecol 1991; 165(pt 1):1731-1737

14. Wachtel S, Elias S, Price J, et al: Fetal cells in the maternal circulation: Isolation by multiparameter flow cytometry and confirmation by polymerase chain reaction. Hum Reprod 1991; 6:1466-1469

15. Shapiro HM: Practical Flow Cytometry, 2d edition. New York, NY, Alan R. Liss, 1988

16. Adinolfi M, Camporese C, Carr T: Gene amplification to detect fetal nucleated cells in pregnant women (Letter). Lancet 1989; 2:328-329

17. Lo YM, Patel P, Wainscoat JS, et al: Prenatal sex determination by DNA amplification from maternal peripheral blood. Lancet 1989; 2:1363-1365

18. Lucas JN, Tenjin T, Straume T, et al: Rapid human chromosome aberration analysis using fluorescence in situ hybridization. Int J Radiat Biol 1989; 56:35-44

19. Lichter P, Cremer T, Tang CJ, Watkins PC, Manuelidis L, Ward DC. Rapid detection of human chromosome 21 aberrations by in situ hybridization. Proc Natl Acad Sci USA 1988; 85:9664-9668

20. Elias S, Price J, Klinger K, et al: Prenatal diagnosis of aneuploidy using fetal cells isolated from maternal blood (Abstr). Am J Hum Genet 1992; 510 (suppl):A4